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EFFECTS OF SOME MECHANICAL FACTORS ON THE ENDOSPORES OF *BACILLUS SUBTILIS*[1]

GEORGES KNAYSI AND HAROLD R. CURRAN

Laboratory of Bacteriology, State College of Agriculture, Cornell University, Ithaca, New York; and Dairy Products Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Washington, D. C.

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ABSTRACT

KNAYSI, GEORGES (Cornell University, Ithaca, N. Y.) AND HAROLD R. CURRAN. Effects of some mechanical factors on the endospores of *Bacillus subtilis*. J. Bacteriol. **82**:691–694. 1961.—Phase contrast microscopy was used to study incipient spore germination and early growth under conditions involving agitation with a platinum wire loop, sonic oscillation, and freezing and thawing. Treatment resulted in change in optical properties and destruction of a certain proportion of the spores. Up to one-tenth of the spores was destroyed by loop agitation. Sonic oscillation had little apparent effect upon incipient germination (darkening) of the spores, but subsequent development involving growth was stimulated in a considerable proportion. The effect of freezing and thawing upon incipient germination is discussed.

Bacteriologists, in general, have an exaggerated concept of the resistance of the bacterial endospore, not only to heat and chemicals, but also to various other factors, including those mechanical in nature.

In the course of an investigation on endospore germination in *Bacillus subtilis* strain 15U, certain observations suggested that the endospore of this highly resistant organism is not so tough physically as might be supposed. Our technique involved the spreading of the spores, as uniformly as possible, on the surface of a cover glass. This often involved vigorous rubbing (with the loop) of a droplet of spore suspension almost until it dries up. Variation in the results led to a careful examination of the resulting film, which gave evidence of mechanical destruction of some of the spores. Mechanical injury to vegetative cells by rubbing with the loop was observed by Yegian and Porter (1944), and vulnerability of the endospore to pressure applied on a cover glass has also been reported (Monk, Hess, and Schenk, 1957; Tomcsik and Baumann-Grace, 1959; Lewis, Snell, and Burr, 1960), but it was surprising that the endospore would also be susceptible to injury by the apparently gentle pressure of the loop. This led to a more systematic study of the effect, not only of rubbing with the loop, but also of sonic vibration and of freezing and thawing on the endospore and its germination.

[1] This study was carried out in the Dairy Products Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture, Washington, D. C.

EXPERIMENTAL METHODS AND RESULTS

Effect of rubbing with the loop. 1) Procedure:—The spore suspension employed was prepared, by dilution with distilled water, from a stock suspension that had been washed 13 times with distilled water, and was free of vegetative cells. A smear was prepared by depositing a loopful of diluted suspension on a cover glass and spreading it into a film with the loop in a warm current of air. This often required almost continuous loop motion until the droplet was nearly dry. The cover glass was then inverted on a hardened film of 2% agar (Difco Laboratories, Detroit, Mich.) and observed in dark contrast with the phase microscope (American Optical Company, Southbridge, Mass.), using an oil immersion objective and a 15× ocular. This preparation was compared with one made by depositing the droplet of suspension directly on the agar film, removing the excess liquid with a capillary glass tube, covering the film with a cover glass, and observing with the phase microscope as above.

2) Results:—The results are recorded in Table 1 and show that the common practice of pre-

TABLE 1. *Effect of rubbing with the loop*

Kind of preparation	Dark spores	Remarks
	%	
Droplet of spore suspension spread with loop on cover glass until dry	6.2; 10.7	There were also many shells. Sometimes whole groups of spores seemed to be disintegrated beyond recognition.
Droplet of spore suspension deposited directly on agar film	1.7; 1.6	Only one shell was seen. The preparation was free of debris.

paring smears of spores by spreading with the loop may destroy a variable proportion of the spores, depending on the extent of rubbing. In our experiments, as many as one-tenth of the spores were destroyed. We did not determine whether or not germination of the remaining, apparently intact spores was in any way affected.

Effect of sonic vibration. The destruction of endospores by exposure to supersonic vibrations at a frequency of 400 kc/sec was reported by Beckwith and Weaver (1936). Berger and Marr (1960) observed a similar effect of sonic vibration at 75 w in a 10-kc oscillator. On the other hand, Heiligman, Desrosier, and Broumand (1956) used ultrasonic vibration (about 102 w, 5 min) to prepare spore suspensions free of vegetative cells. To the authors' knowledge, no information is available on the effect, if any, of sonic vibrations on spore germination.

1) Procedure:—Spores washed eight times with distilled water were resuspended in distilled water. The suspension, usually containing between 2×10^8 and 3×10^8 spores per ml, was subjected to sonic vibration at a frequency of 9.5 kc/sec for periods up to 210 min. At 20-min intervals, samples were taken to study the effect of the treatment on the spore and its germination or growth. The immediate effect on spores was observed with the phase microscope mounted on a thin film or 2% agar as described in the preceding section. Any changes in the optical properties of the spores, as well as the presence of spore debris, were noted. To study the effect on germination or growth, the spores were spread on agar films containing 0.25% glucose and a 0.01 M mixture of mono- and dipotassium phosphate in equimolar proportions, or on a film of brain heart agar (Difco), and incubated at 46 C for 1 and 2 hr, respectively. At the end of the incubation period, incipient germination was judged by the proportion of spores that turned dark when observed in dark contrast with the phase microscope. Growth was considered positive when a dark spore appeared to be enlarged. In one experiment the spores were heated at 88 C for 12 min and the results compared with those obtained with unheated spores.

Incipient germination was observed on the glucose-phosphate agar described above, after it had been found that germination was initiated just as rapidly on this as on the more complex media. For the study of growth, however, the richer medium appeared more desirable.

2) Results:—The exposure of spores to sonic vibrations causes some increase in the proportion of dark spores (Table 2). This proportion reaches a maximum, the level of which may vary from experiment to experiment but most often corresponds to an exposure of 40 min. Other experiments not reported in Table 2 show fluctuations in the proportion of dark spores upon longer exposures, which are explainable by the assumption that dark spores are constantly produced and destroyed by the treatment.

More interesting is the effect on the process of germination. The initial stage, recognized by

TABLE 2. *Effect on sonic vibration*

Period of exposure to vibrations	Dark spores immediately after exposure to vibration	Heating at 88 C for 12 min before incubation	Spores that turned dark upon incubation for 1 or 2 hr at 46 C	Spores showing growth after 2 hr at 46 C	Total dark spores
min	%	%	%	%	%
0	2.5	–	37.5*		
20	3.1	–	36.8*		
40	4.4	–	38.7*		
60	4.0	–	38.3		
0		–	48.2†	17.0	65.2
60		–	30.7†	30.2	60.9
0		+	34.7†	60.3	95.0
60		+	17.8†	77.2	95.0

* Incubated 1 hr on glucose-phosphate agar. The figures are averages from two experiments.

† Incubated 2 hr on brain heart agar.

the change in optical properties (change from bright to dark), does not seem to be significantly affected by exposure to vibration. On the other hand, the subsequent stages, which result in growth and emergence of the germ cell, are significantly stimulated. This is clearly shown (Table 2) both with and without heat activation of the spores before incubation. In both experiments, the total proportion of germinating spores changed little upon exposure to vibration, but a much greater proportion of the exposed than of the unexposed spores reached advanced stages of germination when incubated for 2 hr. in a nutrient medium at 46 C. In the two experiments reported, the differences are about 13% units in spores not activated by heat and 17% units in spores activated by heat before incubation. These differences are explainable by assuming that, besides destroying some of the spores and causing a change in the optical properties of others, vibration also increases the permeability of many spores, without change in their optical properties or injury to their growth mechanism. Thus, growth would be stimulated. However, the fact that the total proportion of dark spores is nearly the same in both exposed and unexposed indicates that this early stage in the germination process is practically unaffected by vibration, perhaps because the site of germination is in a superficial region of the spore and is not so dependent on permeability.

Effect of freezing and thawing. The effect of freezing and thawing on vegetative bacterial cells was studied by Hilliard and Davis (1918), Luyet and Gehenio (1940), Haines (1938), Kyes and Potter (1939), and somewhat more recently by Stille (1950). Hilliard and Davis further noted that the spores of *B. subtilis* showed, in frozen mixtures, greater viability than nonsporulating species. Haines found that rapid freezing had little effect on spores, but slow freezing reduced the proportion of viable spores in *Bacillus mesentericus* to 0.73. The spores of *Bacillus cereus* were apparently not affected by slow freezing. Despite the wide-spread study of spores in recent years, the effects of freezing and thawing on the viability, integrity, and germination of the spore have remained obscure.

1) Procedure:—Spores, freed of vegetative cells and washed 13 times with distilled water, were again suspended in distilled water, making a heavy suspension with creamlike appearance. A small portion of this suspension was diluted with distilled water to a concentration of 8×10^7 spores per ml. This secondary suspension contained 0.66% dark spores, and when mounted on the glucose-phosphate agar described above and incubated for 1 hr at 46 C, gave 41.8% dark spores. The secondary suspension was then divided into three portions: I, C, and L, of 1 ml each. These portions were measured into screw-capped test tubes. Portion I was intermittently frozen and thawed several times a day by placing in a slanted position in the freezing compartment of an electric refrigerator (at about −10 C). It usually froze in 20 to 30 min. Portion C was frozen in a like manner and was kept continuously frozen, except when taken out to be tested. Portion L was kept in the liquid state in the main body of the refrigerator (at about 5 C).

TABLE 3. *Effect of freezing and thawing on dark spores*

Treatment	Before incubation	After incubation at 46 C for 1 hr	Net*
	%	%	%
Untreated suspension	0.7	41.8	41.1
I_{20}	0.8	26.7	25.9
C_1	0.8	39.5	38.7
L	1.1	45.2	44.0
I_{40}	0.3	23.1	22.8
C_2	0.7	34.1	33.4
L	1.3	46.4	45.1
I_{60}	1.6	18.2	16.6
C_3	0.3	26.2	25.9
L	1.1	47.5	46.3
I_{100}	2.6	12.7	10.0
C_4	1.3	24.8	23.5
L	3.2	50.8	47.6

* Column 3 minus column 2.

2) Results:—The results of a typical experiment are recorded in Table 3. It may be readily seen that freezing and thawing at first cause a reduction in the proportion of dark spores initially presented in the suspension. The results with C_1 show that even a single freezing and thawing may bring about such reduction. This is followed by a slow, gradual increase. In the suspension which was not frozen, there is no initial reduction; the proportion of dark spores

steadily increases. The proportion of spores that begins to germinate within 1 hr at 46 C is reduced after the 100th freezing and thawing from an initial 41.1 to 10.0%. The results with suspension C show that even a single freezing and thawing cause an appreciable decrease, so that after the fourth sequence only 23.5% would begin to germinate within 1 hr. As before, suspension L shows a gradual increase in the rate of incipient germination from an initial 41.1 to 47.5%. The experiment ran 6 weeks.

At first, these results appeared to lead to the surprising conclusion that freezing and thawing hinders germination. It was noticed, however, that there was a definite reduction in the turbidity of suspension I when compared with L. By microscopic count there were 4.0×10^7 spores per ml in suspension I and 7.9×10^7 per ml in L, a reduction of 49.4%. Consequently, the correct interpretation of these results must take into consideration not only the possible effect of freezing and thawing on the rate of germination, but also the destructive effect of this treatment on the spores, and any known peculiarity of the organism under study that may have a bearing on the results.

Spores of the strain under investigation are not homogeneous in their tendency to germinate. About half of them germinate readily in a suitable environment, and the remainder germinate very slowly unless heat activated. The results may be explained by assuming that a certain proportion of the readily germinating half are destroyed by every freezing and thawing. This reduces the proportion of this type of spores in the suspension, so that a smaller proportion would show a tendency to germinate after each subsequent treatment. In the present case it appears that, if freezing and thawing had a stimulating effect on germination, the effect is overshadowed by the destructiveness that this treatment had on the spores.

Death by freezing is usually attributed to disruption of cell organization by internal formation of ice crystals. Accordingly, death of the spore by freezing would be evidence for the presence of free water. It is also possible to visualize cell destruction by freezing as a result of the formation of ice crystals around the cell. In this case the spore would be crushed from without.

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